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Award Number: DAMD17-01-1-0020

TITLE: The Role of Steroid Receptor Coactivators in the Development of Prostate Cancer

PRINCIPAL INVESTIGATOR: Jang Hyeon Cho, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

REPORT DATE: September 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2003	Final (15 Aug 2001 - 14 Aug 2003)

4. TITLE AND SUBTITLE

The Role of Steroid Receptor Coactivators in the Development of Prostate Cancer

5. FUNDING NUMBERS

DAMD17-01-1-0020

6. AUTHOR(S)

Jang Hyeon Cho, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baylor College of Medicine
Houston, TX 77030

E-Mail: jcho@bcm.tmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

In prostate cancer, androgen receptor (AR) supervises several key genes expressions. In the cell. AR exerts its regulatory control on a target cell only in the presence of its ligand, androgen. The regulatory functions of AR are more complex and are fine-tuned by accessory proteins. These proteins are required for the maximum biological impact by androgen. These modulators, called coactivators, provide a positive stimulus for receptor action. Our laboratory has cloned the first nuclear receptor coactivator SRC-1. SRC-1 and its related family members, SRC-2 and -3, have the capacity to activate the transcriptional activity of steroid receptor. However, the role of steroid receptor coactivators in prostate cancer is still unclear. To understand the function of these genes in the human prostate cancer, we have performed in situ hybridization on human prostate cancer, and generated SRC-3 overexpressing stable cell lines. During two years of this award, we have examined the SRC-3 is highly expressed in the prostate tumors and its expression is highly correlated with tumorigenesis by regulating the cell proliferation and cell growth.

14. SUBJECT TERMS

Prostate cancer, Steroid receptor coactivator

15. NUMBER OF PAGES

16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Prostate cancer is the most frequently diagnosed solid tumor and the second leading cause of cancer death in man in the United State (1). Cancer initially arises from abnormal expression of certain key genes, which oversee vital cellular processes. In prostate cancer, it is the task of the androgen receptor (AR) to supervise the expression of these key and vital genes in the cell. AR exerts its regulatory control on a target cell only in the presence of its ligand, androgen. In the cell, the regulatory functions of AR are more complex and are fine-tuned by accessory proteins. These accessory proteins are required for the maximum biological impact by androgen. These modulators, called coactivators, provide a positive stimulus for receptor action (2-9). Our laboratory has successfully cloned the first nuclear receptor coactivator, steroid receptor coactivator, SRC-1 (2). Subsequently, the other two family members were cloned later (2, 7, and 8). They all have the capacity to activate the transcriptional activity of steroid receptors. However, the role of steroid receptor coactivators (SRCs) in prostate gland and other reproductive organs is still unclear. It has been shown that SRC-3 is overexpressed and amplified in 60% and 7-8% of breast cancer samples respectively. Also, we have demonstrated that in human breast and prostate cancer cell lines, SRC could enhance the AR transcriptional activity and DNA synthesis. Therefore, we hypothesize that the SRC may play an important role in the initiation and progression of prostate cancer. To test this hypothesis, we have performed 1) *in situ* hybridization on human prostate cancer samples to examine the expression pattern of SRC-3 in prostate cancer, 2) construction of over- and under-expressing vectors for SRC-3 in prostate cell lines to examine its role in cell growth, 3) proliferation and cell growth assay on SRC-3 expressing stable cell line and 4) construction of SRC-3 expression vector to generate SRC-3 overexpressing transgenic mice. Finally, we generated the SRC-3 expressing transgenic mice.

Body

This work was carried out according to the following three specific aims;

Aim 1. Study the expression of steroid receptor coactivators in human prostate cancer samples.

Expression analysis of endogenous steroid receptor coactivators (SRC) in human prostate cancer samples. They will be correlated with each other.

Aim 2. Study the effect of over- and under-expression of steroid receptor coactivator-3 (SRC-3) on prostate cancer cell cycle progression in Cells.

Design and development of *in vitro* models of SRC-3 over-expression in the prostate cancer cell lines. Then analyze the growth properties of these stably transfected cell lines that over-expressed SRC-3.

Aim 3. Evaluate the role of steroid receptor coactivator-3 (SRC-3) in prostate cancer progression in vivo.

We propose to generate transgenic mouse expressing SRC-3 in prostate epithelial cells under the control of the prostate specific antigen (PSA) promoter. This promoter has been shown to be highly specific to direct its target to the lateral prostate. The transgenic mouse obtained will then be induced to produce prostate tumors by mating with murine strains exhibiting susceptibility to prostate cancer. The ability of SRC-3 to activate androgen receptor will be examined in correlation with the tumor growth in these animals.

During the first and second years' work of this award, we have accomplished the following subjects;

1. Expression analysis of SRC expression in human prostate tumor samples

We have successfully finished this part of aim and reported already. *In situ* hybridization has been used to study the expression of SRC-3 and its correlation with the tumor stages. Briefly, 1) approximately 47% of prostate tumor samples overexpressed SRC-3 in the tumor area (n=134) but only 8.2% were observed SRC-3 expression in the adjacent normal areas (n=61). 2) SRC-3 expression level was highly correlated with the Gleason score and tumor stages. In addition, SRC-3 over expression is most prominent in androgen resistant tumors. All these results suggest that SRC-3 is an excellent marker for late stage of prostate cancer (please refer 1st annual report of this award).

2. Construction and analysis of SRC-3 over-expressing vector

One of the goals of this work is to construct expression plasmids for coactivators to generate stable cell lines which overexpress SRC-3. We had successfully generated SRC-3 overexpressing cell lines by using RU486 inducible system (11, 12) and confirmed the stable cell lines with several antibodies such as anti-SRC-3 and anti-hemagglutinin (HA) (please refer 1st annual report of this award).

After characterized the SRC-3 stable cell lines, we examined the effect of SRC-3 overexpression in this cell line, especially on the activities of cell proliferation and properties of cell growth. The cell lines were cultured in RPMI 1640 medium which containing 10% fetal bovine serum supplemented with 100U/ml penicillin and 100□g streptomycin. Cells were maintained as monolayer in a 5% CO_2 humidified atmosphere at 37°C and passaged at confluence by trypnization. Hormone-deprived medium was prepared by addition of 10% charcoal-stripped serum instead of untreated whole FBS to the medium. Hormone deprivation was initiated by plating LNCaP cells at about 30% confluence in serum- and phenol red-free medium for 48 hours. Then cells were continuously cultured in stripped medium in the presence or absence of 2nM R1881 for at least 48 hours before further drug treatment.

By using the SRC-3 stable cell lines, first, we examined the role of SRC-3 on the cell proliferation. 3H-thymidine incorporation assay was performed in either presence or absence of steroid hormone R1881. 0.5nCi/well of 3H-thymidine was used in the assay for 16 hours. 3H-thymidine incorporation were measured by liquid scintillation counter. As shown in Figure 1, SRC-3 overexpressing LNCaP cells have 2 times higher rate of 3H-thymidine incorporation when compare to the normal LNCaP cells in the same experimental condition (Fig. 1, bar 1 and 3). And this higher incorporation was decreased by adding Wortmannin, an inhibitor of phosphatidyinositol-3-kinase (PI3K) (Fig. 1, bar 2 and 4). This result suggests that SRC-3 may play an important role in cell proliferation of prostate cancer via PI3K dependent pathway.

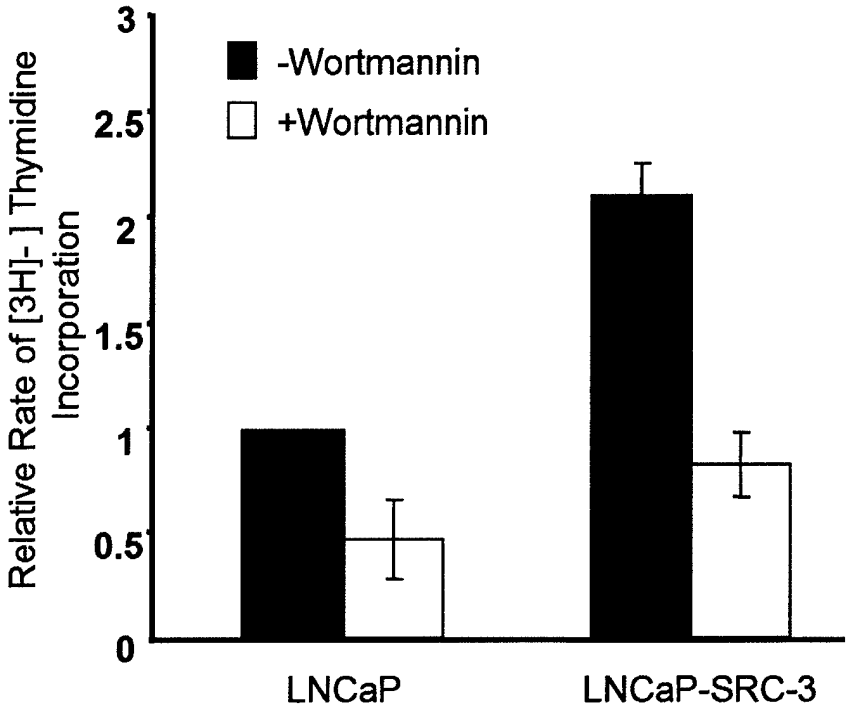

Figure 1. 3H-Thymidine incorporation rate of LNCaP SRC-3 stable cell line

We also determine the cell proliferation rate in the presence or absence of hormone R1881 (Fig. 2). The proliferative activity of hormone dependant cell line LNCaP are gradually increased in days of culture in the presence of hormone R1881 (Fig. 2B, red line) but not increased in the absence of hormone (Fig. 2A, red line). However, proliferative activity of LNCaP SRC-3 stable cell line was up to 2 time higher in the absence of hormone (Fig. 2A, blue line) and was also higher in the presence of hormone (Fig. 2B, blue line), indicating that SRC-3 induced cell proliferation may contribute to the tumor growth of prostate tumor that over express SRC-3. In addition, SRC-3 may also contribute to the growth of hormone independent prostate tumors.

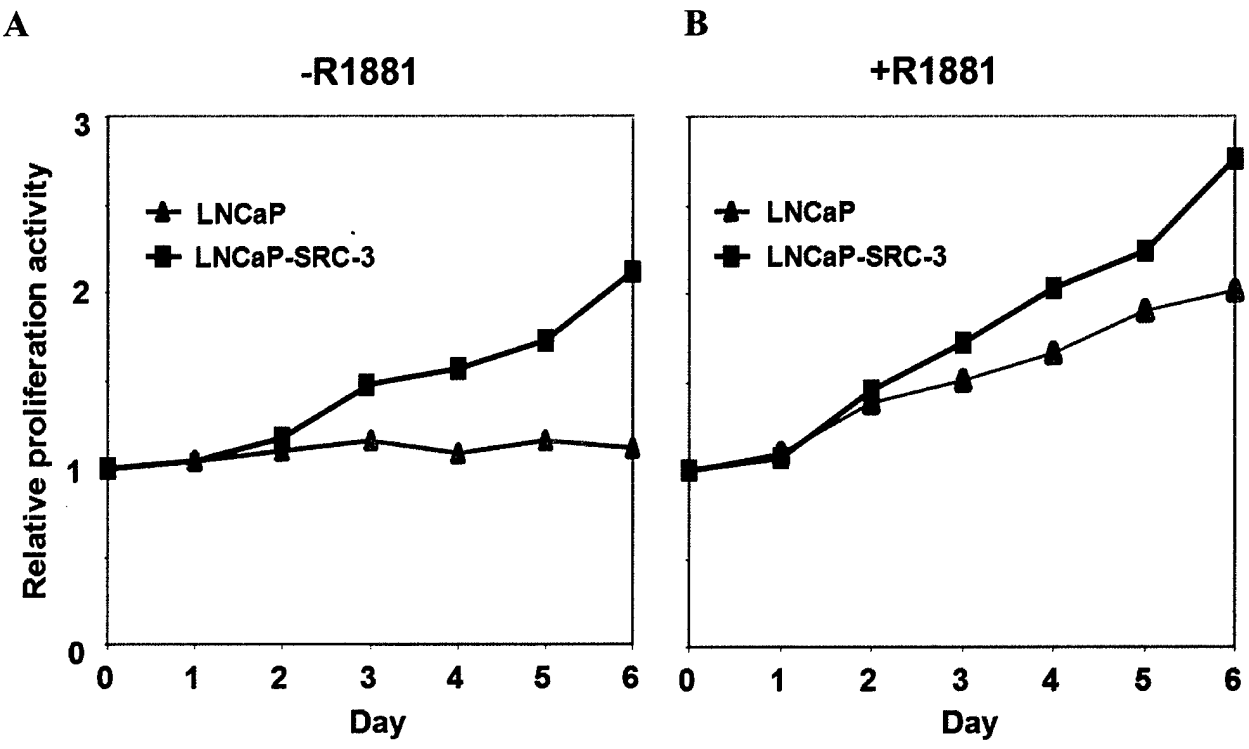

Figure 2. Time course of of NLCaP-SRC-3 proliferation activity in the absence or presence of hormone condition.

To examine the effect of SRC-3 overexpression on the cell morphology, we induced the SRC-3 expression with 10^{-8}M RU486 for 24 hours. As shown in Figure 3, interestingly, we observed that cell size of RU486 treated cells were increased significantly under the phase contrast microscope (Figure 3).

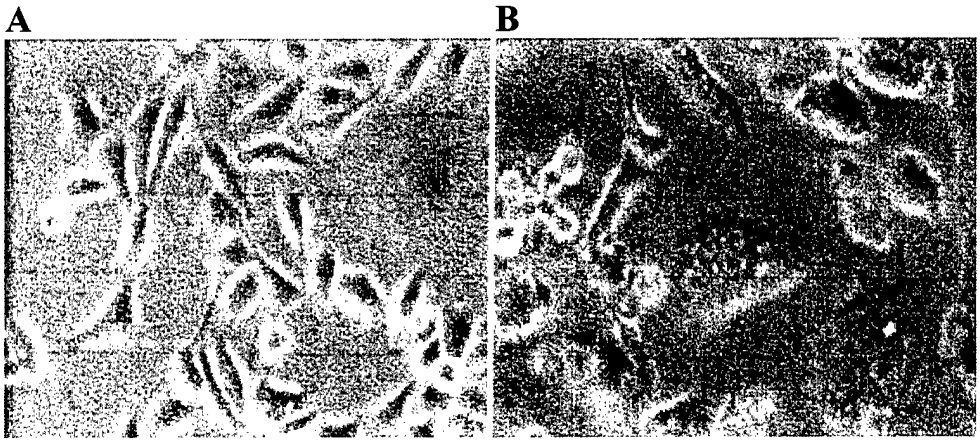

Figure 3. RU486 induced morphological changes in LNCaP SRC-3 stable cell line. A: without RU486; B: treated with 10^{-8}M RU486

To analyze this effect further, we determined the DNA content and cell size in detail using a Becton Dickinson FACS Calibur flow cytometer with cell Quest software. For FACS analysis, 10,000 of single cell were collected from each RU486 treated and untreated cell groups, and calculated the DNA content of G1 population and the forward scatter height parameter (FSC-H) of those cells.

Consistent with our previous results, RU486 treatment led to a rightward shift in the mean of FSH-H histogram of G1 phase LNCaP-SCR-3 cells significantly ($p<0.005$) from untreated LNCaP cells (compare Figure 4B, blue arrow to red arrow). Same experiment was performed in the parental control LNCaP cell line, as expected, we could not detect any rightward shifts in these parental LNCaP cells (Figure 4A, compare blue arrow to red arrow). Taken together, our results suggest that overexpression of SRC-3 promotes cell growth to increase cell size.

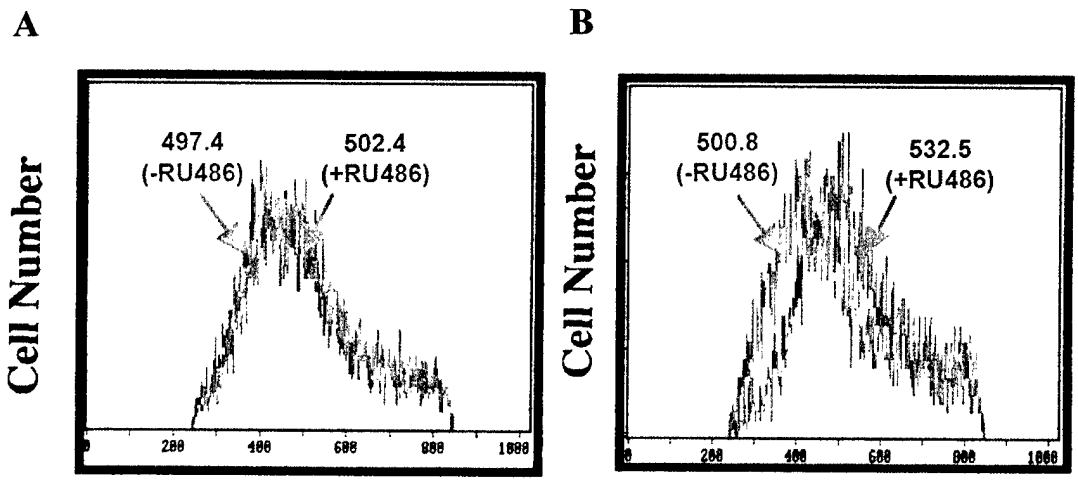

Figure 4. Forward scatter height (FSC-H) analysis of LNCaP parental cell line and SRC-3 stable cell line.

3. Generation of transgenic mouse line expressing SRC-3

We have tried to generate transgenic mice with SRC-3 overexpression in the prostate. However, we were not successful due to the overexpression of SRC-3 is detrimental to the health of animals. To overcome this difficulty, we adopted an inducible system to express SRC-3. This system includes two plasmids: a regulator and a target (Fig. 5). The regulator is a fusion protein consisting of three functional domains: a DNA-binding domain derived from the Gal4 DBD, a p65 transactivation domain, and a mutant progesterone receptor (PR) ligand-binding domain that binds to mifepristone (RU486) but not to endogenous ligands (Fig. 5A). The target construct contains *SRC-3* cDNA placed under the control of a TATA minimal promoter and 4 copies of 17mer Gal4 DNA binding sites (Fig.5C). In the absence of RU486, the regulator is not active. When RU486 is administered, it binds to the regulator and induces its conformational change, resulting in activation of the regulator, which subsequently binds to the Gal4 DNA binding sites and activates *SRC-3* expression. We have generated transgenic mice using probasin

promoter to express the regulator specifically in the prostate. PCR result in Figure 5B demonstrates that we have successfully generated regulator mice (Fig. 5B). Similarly, we have also generated target SRC-3 mice.

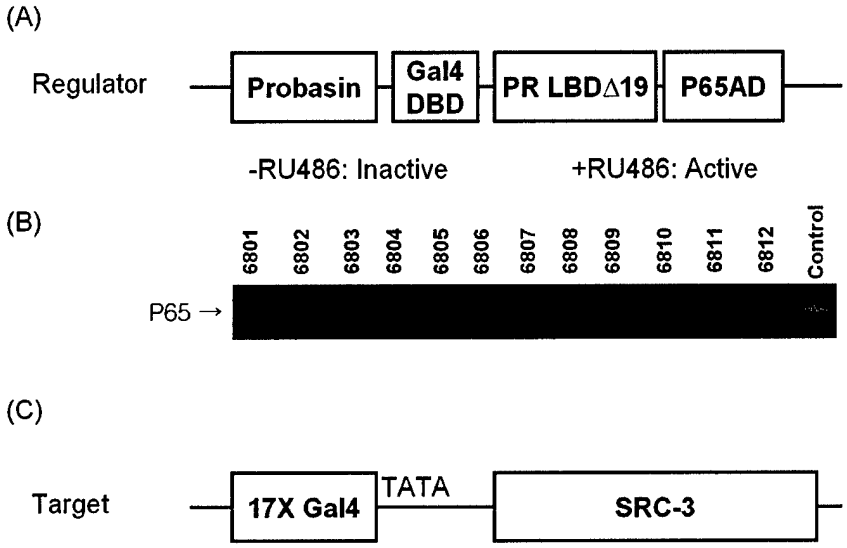

Figure 5. A Schematic drawing and a PCR result of a regulator (A) and a target constructs (C) for specific expression of SRC-3 in the prostate gland. (B) PCR result with P65 primers.

We are currently mating these two mice to generate inducible SRC-3 expression in the prostate. In the next few years, we will characterize these mice and cross them to prostate tumor model (TRAMP mice) to study the effect of overexpression of SRC-3 on prostate tumorigenesis as proposed in subaims 3b and 3c.

Key Research Accomplishments:

1. We detected SRC-3 overexpression in human prostate tumors but not in the adjacent non-tumor area.
2. We found SRC-3 level is highly correlated with Gleason score and tumor stages.
3. We have successfully established an RU486 inducible expression system in prostate cell lines.
4. We found that SRC-3 mediated cell proliferation is dependant on PI3K pathway.
5. We found that SRC-3 can modulate the cell proliferation in a steroid hormone independent manner.
6. We found that overexpression of SRC-3 promotes cell growth to increase cell size.
7. We have constructed transgenic expressing construct for SRC-3 in the prostate gland.
8. We have successfully generated regulator mice.
9. We have generated target SRC-3 mice.

Reportable Outcomes

1. We have established the RU486 inducible expression system in prostate cell lines.
2. We have generated SRC-3 overexpressing stable cell line.
3. We have found that the role of SRC-3 as an important modulator for cell proliferation and growth.
4. We have generated regulator mice and target SRC-3 transgenic mice.
5. Because of the support of this award, I have opportunities to be trained as a postdoctoral fellow in Dr. Ming-Jer Tsai's laboratory in Baylor College of Medicine during past two years. This training will contribute my personal and professional career.

Conclusions

During past two years' work of this award, entitled in "The role of steroid receptor coactivators in the developmental cancer", we have accomplished several important results as presented in above. Briefly, SRC-3, one of steroid receptor coactivators, is overexpressed in prostate cancer patients, especially in tumor area of prostate cancer (47%). Also, SRC-3 overexpression is correlated with the increasing severity of prostate cancer. And we have successfully established a SRC-3 inducible stable cell lines. The analysis of this stable cell line revealed that SRC-3 is an important modulator for cell proliferation and cell growth.

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Appendices

- Figures and tables are embedded in the text.